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RESEARCH ARTICLE

9-(4'-dimethylaminophenyl)-2,6,7-trihydroxy-xanthene-3-one is a Potentially Novel Antiplatelet Drug which Antagonizes the Effect of Thromboxane A_2

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Abstract: ***Background*:** Currently, used oral antiplatelet drugs are both limited and associated with the risk of treatment failure/resistance. Research in this area is hence highly desired. A series of xanthene-3-ones derivatives, we had synthesized, showed us that these derivatives had antiplatelet activity. As far as we know, no research on the effects of xanthen-3-ones in this area has been done.

***Objective*:** The aim was to study the antiplatelet potential of a series of synthesised 9-phenyl-xanthene-3-ones and to find the ideal structural feature(s) for antiplatelet potential and determine the mechanism of action.

***Methods*:** The compounds were synthesized from 1,2,4-triacetoxybenzene and various benzaldehydes. The reaction proceeded smoothly under acidic alcoholic conditions, furnishing the desired products in good yields. The compounds were first screened in whole human blood where platelet aggregation was induced by arachidonic acid. Further analysis was targeted at search of the mechanism of action.

***Results*:** Initial screening showed that a majority of the synthesized derivatives had substantial antiplatelet potential. None of the compounds were able to block cyclooxygenase 1 or thromboxane synthase. The mechanism appeared to be based on antagonism of thromboxane effects. The most potent compound 9-(4'-dimethylaminophenyl)-2,6,7-trihydroxy-xanthene-3-one had better potential to block collagen induced platelet aggregation than clinically used acetylsalicylic acid.

***Conclusion*:** The last mentioned derivative is promising for further *in vivo* testing.

Keywords: Platelet, xanthene-3-one, thromboxane, antiplatelet, cyclooxygenase, arachidonic acid.

1. INTRODUCTION

Finding, testing and describing the mechanism of action of new substances that block platelet aggregation is challenging. Current oral antiplatelet drugs include acetylsalicylic acid (ASA), an irreversible antagonist of cyclooxygenase-1 (COX-1), and antagonists of ADP receptors P2Y12 (clopidogrel, prasugrel, ticagrelor, elinogrel and cangrelor) Treatment with both types of substances is problematic: in around 5-45% of ASA users there is a treatment failure and/or resistance [1] and antagonists to ADP receptors (ticlopidin and clopidogrel) are associated with drug interactions [2, 3]. The latest representatives of the latter (ticagrelor, elinogrel, cangrelor) are not prodrugs and hence have a smaller potential for such interactions but their use in clinical practise is relatively recent and the side-effects of long-term treatment have not yet been established. Consequently, discovery of new substances with antiplatelet potential on other aggregations levels is desired. Importantly, new drugs acting at different levels of aggregation than the targets of the currently used

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oral anti-platelet drugs could overcome the complex issues with the drugs used.

Platelet aggregation is a complex process which is still not fully understood. There are many proaggregatory stimuli. The key mediator appears to be thromboxane A_2 (TXA_2), which is synthetized in platelets on stimulation by various aggregation inducers including ADP, collagen and thrombin. TXA_2 formation is initiated by release of arachidonic acid (AA) from the cytoplasmatic membrane, followed by its conversion by platelet COX-1 into prostaglandin H_2 and into TXA_2 via platelet thromboxane A_2 synthase. Beyond proaggregatory activity, TXA_2 is also a potent vasoconstrictor [4]. Moreover, platelet thromboxane A_2 receptors are increased in patients with acute myocardial infarction [5].

Synthesis of xanthene-3-ones (9-Aryl-6-hydroxy-3*H*-xanthen-3-one) as fluorescent sensors of divalent ions [6] and the antioxidant properties of another synthesized xanthene-3-ones have been established [7], however up to now, the potential use of xanthene-3-ones is limited. Two compounds, 9-[1-(2-methyl-4-methoxyphenyl)]-6-hydroxy-3H-xanthen-3-one (Tokyo green) and another fluorescein derivate 9-[1-(4-tert-butyl-2-methoxyphenyl)]-6-hydroxy-3H-xanthen-3-one (Granada green) are sensitive dyes for intracellular phosphate detection [8, 9], 3',6'-bis(4-guanidinobenzoyloxy)-5-(N'-4-carboxylphenyl)thioureidospiro[isobenzofuran-1(3H), 9'-[9H]xanthen]-3-one is useful for the detection of plasminogen activator activity [10] and 3',6'-bis(diethylamino)-2-(pyridine-2-ylmethyl)spiro[isoindoline-1,9'-xanthen]-3-one is highly efficient for the recognition and determination of Hg^{2+}. [11] Research has also been targeted to xanthene-9-ones. Two of these compounds showed anticancer properties. 1-hydroxy-3-(2´-hydroxy-3´-(butylamino)propoxy) xanthone acts as a topoisomerase IIα inhibitor[12] and 4-[(2E)-3,7-dimethoxyocta-2,6-dien-1yl]-1,5,8-trihydroxy-3-methoxy-9*H*-xanthen-9-one exhibited antiproliferative activity against leukemia cells [13]. Some xanthen-9-ones were found to be non-specific and highly active inhibitors of isolated COX-1 and COX-2 enzymes [14]. As far as we know, no research on the effects of xanthen-3-ones toward platelets or COX enzymes has been conducted.

A series of xanthene-3-ones derivatives was synthetized in an effort to find novel metal chelators. Interestingly, during screening assays, we found that these derivatives had antiplatelet activity. This study was aimed to synthesize and investigate the antiaggregatory properties of newly synthetized xanthene-3-one derivatives, to find ideal structural feature(s) for antiplatelet potential and to determine their mechanism of action.

2. MATERIAL AND METHODS

2.1. Chemistry

2.1.1. Synthesis of Xanthene-3-on Derivatives

Derivatives of xanthene-3-ones were prepared according to a known procedure which includes two-fold Friedel-Crafts alkylation of 1,2,4-triacetoxybenzene reflux in ethanol and sulphuric acid with addition of different benzaldehydes and potassium peroxodisulphate as an oxidizing agent. [15, 16] In detail, a round-bottom flask equipped with a condenser and mechanical stirrer was filled with 1,2,4-triacetoxybenzene (5 g) and 50% EtOH (75 mL). Concentrated sulphuric acid (3 mL) was added and the white suspension was heated to reflux, resulting in a clear, honey colored solution. Various benzaldehydes (10 mmol) were added dropwise to the mixture over a 2 min period. The stirred mixture was kept at reflux for another 60 min. Potassium peroxodisulphate (2,70 g) was then gradually added at 80 °C over a period of 50 min. The contents were brought to reflux for another 20 min and then poured onto ice water. Obtained red crystals were washed with water and later dried in vacuum at 60 °C.

2.1.2. General Methods.

Microanalyses for C, H and N were performed on a Perkin Elmer 2400 elementary analyzer (Germany). The IR spectra of the synthesized compounds were recorded by Shimadzu IR Prestige 21 ID using KBr pellets. The 1H and 13C nuclear magnetic resonance spectra were recorded at 600 and 150 MHz, respectively, in deuterated dimethyl sulfoxide (DMSO-d_6) at 25 °C using the NMR spectrometer Bruker AV600, with tetramethylsilane as internal reference. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. Electrospray ionization mass spectrometry (ESI-MS) measurements were performed on a high performance liquid chromatography-mass spectrometry (HPLC-MS triple quadrupole 6420 instrument equipped with an autosampler (Agilent Technologies, Palo Alto, CA, USA). The desolvation gas temperature was 300 °C with a flow rate of 6.0 L min-1. The fragmentor voltage was 135 V and the capillary voltage was 4.0 kV. The mobile phase was 0.1% formic acid in 50% methanol and the flow rate of the mobile phase was 0.2 mL min-1. Mass spectra as total ion current spectra were recorded in *m/z* segment of 10-2250. All data acquisition and processing were performed using Agilent MassHunter software. The purity of the final compounds was ≥99%.

2.1.3. 9-(2'-hydroxy-5'-bromophenyl)-2,6,7-trihydroxyxanthene-3-one (1).

Red crude material; yield 78.4%; m.p. 203 °C; IR (KBr) λmax/cm-1: 3600-2500 (phenol OH); 750 (C-Br); 1650 (C=O); 860 (substituted benzene); 1600 (C=O); 1300 (C-O); 1200 (phenol OH); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 163.3 (C-12, C-13), 154.2 (C-6'), 152.5 (C-2, C-7), 147.6 (C-3, C-6), 146.3 (C-9), 133.6 (C-4'), 132.4 (C-2'), 122.4 (C-1'), 118.4 (C-5'), 116.0 (C-11, C-14), 110.3 (C-3'), 107.4 (C-1, C-8), 102.2 (C-4, C-5); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 10.19 (2H, br. s., OH), 9.90 (2H, br. s., OH), 7.63 (1H, dd, H-4', J_1 = 8.3 Hz, J_2 = 2.0 Hz), 7.44 (1H, d, H-2', J = 2.8 Hz), 7.09 (1H, d, H-5', J = 9.1 Hz), 6.95 (2H, s, H-4, H-5), 6.55 (2H, s, H-1, H-8); ESI-MS (m/z): 417.1 $(M+H)^+$; 414.9 $(M-H)^-$ Elemental analysis for $C_{19}H_{11}O_6Br$ (415.9): $\%C_{calculated}$. 54.82; $\%H_{calculated}$. 2.64: $\%C_{found}$: 54.86; $\%H_{found}$ 2.61; purity: 99.95%.

2.1.4. 9-(2'-hydroxy-3'-methoxyphenyl)-2,6,7-trihydroxyxanthene-3-one (2)

Cadmium red crude material; yield 86.7%; m.p. 178 °C; IR (KBr) λmax/cm-1: 3400-2400 (henol OH); 850 (substituted benzene); 1600 (C=O); 1300 (C-O); 1450 (O-CH_3); ^{13}C

NMR (150 MHz, DMSO-d_6, δ/ppm): 163.0 (C-12, C-13), 152.6 (C-2, C-7), 149.8 (C-9), 148.0 (C-5'), 147.5 (C-3, C-6), 143.7 (C-6'), 121.6 (C-2'), 120.2 (C-1'), 119.5 (C-3'), 116.2 (C-11, C-14), 112.8 (C-4'), 107.9 (C-1, C-8), 102.1 (C-4, C-5), 55.9 (OCH_3); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.91 (3H, br. s., OH), 9.13 (1H, br. s., OH), 7.23 (1H, dd, H-4', J_1 = 8.2 Hz, J_2 = 1.5 Hz), 7.05 (1H, t, H-3', J = 7.9 Hz), 7.00 (2H, s, H-4, H-5), 6.80 (1H, dd, H-2', J_1 = 7.6 Hz, J_2 = 1.5 Hz), 6.60 (2H, s, H-1, H-8), 3.92 (3H, s, OCH_3). ESI-MS (m/z): 367 $(M+H)^+$ Elemental analysis for $C_{20}H_{14}O_7$ (366): $\%C_{calculated}$. 65,60; $\%H_{calculated}$. 3.80: $\%C_{found}$: 65.51; $\%H_{found}$ 3.84; purity: 98.80%.

2.1.5. 9-(3',4'-dihydroxyphenyl)-2,6,7-trihydroxy-xanthene-3-one (3)

Red crude material; yield 82.2%; m.p. 203-205 °C; IR (KBr) λmax/cm-1: 3600-2500 (phenol OH); 750 (C-Br); 1650 (C=O); 860 (substituted benzene); 1600 (C=O); 1300 (C-O); 1200 (phenol OH); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 152.4 (C-12, C-13), 151.9 (C-2, C-7), 147.5 (C-3, C-6), 147.4 (C-9), 147.1 (C-4'), 145.5 (C-5'), 123.6 (C-1'), 121.1 (C-2'), 116.9 (C-6'), 115.9 (C-3', C-11, C-14), 108.3 (C-1, C-8), 102.1 (C-4, C-5); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.55 (5H, br. s., OH), 7.04 (1H, d, H-3', J = 8.1 Hz), 7.01 (2H, s, H-4, H-5), 6.87 (1H, d, H-6', J = 2.1 Hz), 6.86 (2H, s, H-1, H-8), 6.77 (1H, dd, H-2', J_1 = 7.9 Hz, J_2 = 1.9 Hz); ESI-MS (m/z): 353.3 $(M+H)^+$ Elemental analysis for $C_{19}H_{12}O_7$ (352): $\%C_{calculated}$. 64.80; $\%H_{calculated}$. 3.41: $\%C_{found}$: 64.58; $\%H_{found}$ 3.35; purity: 99.72%

2.1.6. 9-(3',4'-dimethoxyphenyl)-2,6,7-trihydroxy-xanthene-3-one (4)

Crude material; yield 68.5%; m.p. 168-169 °C; IR (KBr) λmax/cm-1: 3400-2500 (phenol OH); 865 (substituted benzene); 1600 (C=O); 1300 (C-O); 1200,1250 (phenol OH); 1400 (O-CH_3); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 152.0 (C-2, C-7), 149.5 (C-4'), 149.3 (C-5'), 147.4 (C-3, C-6), 146.8 (C-9), 125.7 (C-1'), 121.9 (C-2'), 115.3 (C-11, C-14), 113.0 (C-6'), 111.8 (C-3'), 107.0 (C-1, C-8), 102.2 (C-4, C-5), 55.8 (OCH_3-5'), 55.6 (OCH_3-4'). Signals of atoms C-12 and C-13 were not detected; ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.44 (2H, br. s., OH), 7.22 (1H, d, H-3', J = 8.1 Hz), 7.04 (1H, d, H-6', J = 1.8 Hz), 6.97 (1H, dd, H-2', J_1 = 8.1 Hz, J_2 = 1.8 Hz), 6.74 (2H, s, H-4, H-5), 6.55 (2H, s, H-1, H-8), 3.89 (3H, s, OCH_3-4'), 3.79 (3H, s, OCH_3-5'); ESI-MS (m/z): 379.0 $(M-H)^-$; 381.3 $(M+H)^+$ Elemental analysis for $C_{21}H_{16}O_7$ (380): $\%C_{calculated}$. 66.32; $\%H_{calculated}$. 4.21: $\%C_{found}$: 66.40; $\%H_{found}$ 4.28; purity: 99.85%.

2.1.7. 9-(3',5'-dimethoxy-4'-hydroxyphenyl)-2,6,7-trihydroxyxanthene-3-one (5)

Crude material; yield 74%; m.p. 203 °C; IR (KBr) λmax/cm−1: 3500-2500 (phenol OH); 1600 (C=O); 1300 (C-O); 1200(def.vibr.OH); 1260 (O-CH_3); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 148.1 (C-3' and C-5'), 145.1 (C-9), 136.2 (C-7, C-6), 123.2 (C-1'), 107.0 (C-1, C-8, C-2' i C-6'), 102.2 (C-4 i C-5), 56.2 (OCH_3); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.36 (2H, br. s., OH), 8.80 (1H, br. s., OH), 6.73 (2H, s, H-4, H-5), 6.69 (4H, m, H-1, H-8, H-2' ,H-6'), 3.71 (6H, s, OCH_3-3', OCH_3-5'); ESI-MS (m/z): 397.4 $(M+H)^+$ Elemental analysis for $C_{21}H_{16}O_8$ (396): $\%C_{calculated}$. 63.60; $\%H_{calculated}$. 4.04: $\%C_{found}$: 63.40; $\%H_{found}$ 4.12; purity: 99.72%.

2.1.8. 9-(3'-methoxy-5'-nitro-4'-hydroxyphenyl)-2,6,7-trihydroxy-xanthene-3-one (6)

Crude material; yield 79.2%; m.p. 214-217 °C; IR (KBr) λmax/cm−1: 3700-2400 (phenol OH); 1600 (C=O); 1300 (C-O); 1200 (def.vibr. OH); 1250 (O-CH_3); 1380,1500 (NO_2); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 164.0 (C-12, C-13), 152.5 (C-2, C-7), 149.8 (C-5'), 147.7 (C-3, C-6), 145.4 (C-9), 142.8 (C-4'), 137.4 (C-3'), 123.6 (C-1'), 116.9 (C-6'), 116.7 (C-2'), 115.6 (C-11, C-14), 107.0 (C-1, C-8), 102.3 (C-4, C-5), 57.0 (OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 10.96 (2H, br. s., OH), 9.63 (2H, br. s., OH), 7.54 (1H, d, H-2', J = 1.95 Hz), 7.39 (1H, d, H-6', J = 1.56 Hz), 6.81 (2H, s, H-4, H-5), 6.56 (2H, s, H-1, H-8), 3.92 (3H, s, OCH_3); ESI-MS (m/z): 412.3 $(M+H)^+$ Elemental analysis for $C_{20}H_{13}O_9N$ (411): $\%C_{calculated}$. 58.40; $\%H_{calculated}$. 3.16: $\%C_{found}$: 58.52; $\%H_{found}$ 3.01; purity: 99.73%.

2.1.9. 9-(4'-ethoxyphenyl)-2,6,7-trihydroxy-xanthene-3-one (7)

Red crude material; yield 82.4%; m.p. 216 °C; IR (KBr) λmax/cm-1: 3400-2500 (phenol OH); 3550 (OH); 800 (substituted benzene); 1600 (C=O); 1300 (C-O); 1200 (def.vibr. OH); 1270(ether R-O-C_2H_5); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 159.1 (C-4'), 152.0 (C-2 ,C-7), 147.4 (C-3, C-6), 146.6 (C-9), 130.8 (C-2', C-6'), 125.3(C-1'), 115.3 (C-11, C-14), 114.6 (C-3', C-5'), 106.9 (C-1, C-8),102.2 (C-4, C-5), 63.3 ($O\underline{CH_2}CH_3$), 14.6 ($OCH_2\underline{CH_3}$); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.43 (3H, br. s., OH), 7.36 (2H, d, H-2', H-6', J = 8.6 Hz), 7.19 (2H, d, H-3', H-5', J = 8.6 Hz), 6.73 (2H, s, H-4, H-5), 6.50 (2H, s, H-1, H-8), 4.17 (2H, q, $O\underline{CH_2}CH_3$, J = 6.9 Hz), 1.41 (3H, t, $OCH_2\underline{CH_3}$, J = 6.9 Hz); ESI-MS (m/z): 365.3 $(M+H)^+$ Elemental analysis for $C_{21}H_{16}O_6$(364): $\%C_{calculated}$. 69.23; $\%H_{calculated}$. 4.40: $\%C_{found}$: 69.12; $\%H_{found}$ 4.26; purity: 99.75%.

2.1.10. 9-(4'-dimethylaminophenyl)-2,6,7-trihydroxy-xanthen-3-one (8)

Crude material; yield 65.3%; m.p. 199-203 °C; IR (KBr) λmax/cm−1: 3400-2400 (phenol OH); 834 (*para*-substituted benzene); 1500 (C=O); 1300 (C-O); 1200 (OH def.vibr); 1440 (CH_3-N); 1265 (C-N); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 160.9 (C-12, C-13), 158.0 (C-9), 152.8 (C-2, C-7), 151.5 (C-4'), 147.3 (C-3, C-6), 131.6 (C-2', C-6'), 119.0 (C-1'), 116.1 (C-11, C-14), 111.7 (C-3', C-5'), 109.6 (C-1, C-8), 102.2 (C-4, C-5), 39.8 (NCH_3); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.96 (1H, br. s., OH), 7.44 (2H, d, H-2', H-6', J = 8.5 Hz), 7.23 (2H, s, H-4, H-5), 7.11 (2H, s, H-1, H-8), 7.03 (2H, d, H-3', H-5', J = 8.9 Hz), 3.10 (6H, s, CH_3); ESI-MS (m/z): 366.4 $(M+H)^+$ Elemental analysis for $C_{21}H_{17}O_5N$ (365): $\%C_{calculated}$. 69.40; $\%H_{calculated}$. 4.68: $\%C_{found}$: 69.61; $\%H_{found}$ 4.46; purity: 99.50%.

2.1.11. 9-(4'-trifluormethylphenyl)-2,6,7-trihydroxy-xanthene-3-one (9)

Crude material; yield 69.4%; m.p. 212-214 °C; IR (KBr) λmax/cm−1: 3400-2500 (phenol OH); 820 (*para*-substituted benzene); 1600 (C=O); 1300 (C-O); 1200 (def.vibr. OH); 1030 (C-F); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 151.6

(C-2, C-7), 147.8 (C-3, C-6), 144.2 (C-9), 138.2 (C-1'), 130.3 (C-2', C-6'), 129.5 (C-4', q, $J_{C,F}$ = 31.8 Hz), 125.7 (C-3', C-5', q, $J_{C,F}$ = 3.6 Hz), 124.1 (CF_3, q, $J_{C,F}$ = 272.0 Hz), 115.0 (C-11 , C-14), 106.2 (C-1, C-8), 102.5 (C-4 , C-5). Signals of atoms C-12 and C-13 were not detected; ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.51 (2H, br. s., OH), 8.03 (2H, d, H-3', H-5', *J* = 8.3 Hz), 7.71 (2H, d, H-2', H-6', *J* = 8.3 Hz), 6.75 (2H, s, H-4, H-5), 6.31 (2H, s, H-1,H-8); ESI-MS (m/z): 389.3 $(M+H)^+$ Elemental analysis for $C_{20}H_{11}O_5F_3$ (388): $\%C_{calculated}$. 61.86; $\%H_{calculated}$. 2.83: $\%C_{found}$: 61.91; $\%H_{found}$ 2.64; purity: 99.76%.

2.1.2. 9-(4'-acetamidophenyl)-2,6,7-trihydroxy-xanthene-3-one (10)

Crude material; yield 81.6%; m.p. 209 °C; IR (KBr) λmax/cm-1: 3400-2500 (phenol OH); 800 (para-substituted benzene); 1600 (C=O); 1300 (C-O); 1200,1250 (def.vibr. OH); 1420 (COO^-); 1450-1500 (C-N, NH_2 def.vibr); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 160.9 (C-12, C-13), 157.0 (C-9), 152.9 (C-2, C-7), 148.7 (C-4'), 147.4 (C-3, C-6), 132.0 (C-2', C-6'), 121.0 (C-1'), 116.1 (C-11,C-14),115.2 (C-3',C-5'),109.6 (C-1, C-8), 102.3(C-4,C-5); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 10.40 (2H, br. s., OH), 7.36 (2H, d, H-2',H-6', J = 8.5 Hz), 7.22 (2H, s, H-4, H-5), 7.08 (2H, s, H-1, H-8), 7.02 (2H, d, H-3', H-5', J = 8.3 Hz); Elemental analysis for $C_{20}H_{13}O_7N$ (379): $\%C_{calculated}$. 63.32; $\%H_{calculated}$. 3.43: $\%C_{found}$: 63.41; $\%H_{found}$ 3.25; purity: 99.73%.

2.1.13. 9-(3'-bromophenyl)-2,6,7-trihydroxy-xanthene-3-one (11)

Crude material; yield 78.86%; m.p. 176-179 °C; IR (KBr) λmax/cm−1: 3400-2500 (phenol OH); 800 (*meta*-substituted benzene); 1600 (C=O); 1300 (C-O); 1200,1230 (phenol OH); 750 (C-Br); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 163.2 (C-12, C-13), 152.4 (C-2, C-7), 149.2 (C-9), 147.7 (C-3, C-6), 135.6 (C-5'), 132.4 (C-4'), 131.5 (C-6'), 131.0 (C-3'), 128.3 (C-2'), 122.1 (C-1'), 115.7 (C-11, C-14), 107.2 (C-1, C-8), 102.3 (C-4, C-5); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 7.88 (1H, d, H-4', *J* = 7.5 Hz), 7.75 (1H, t, H-6', *J* = 1.6 Hz), 7.65 (1H, t, H-3', *J* = 7.7 Hz), 7.51 (1H, dd, H-2', J_1 = 8.2 Hz, J_2 = 0.9 Hz), 6.97 (2H, s, H-4, H-5), 6.52 (2H, s, H-1, H-8). Signals of OH groups were not detected. ESI-MS (m/z): 401.3 $(M+H)^+$ Elemental analysis for $C_{19}H_{11}O_5Br$ (400): $\%C_{calculated}$. 57.00; $\%H_{calculated}$. 2.75: $\%C_{found}$: 57.31; $\%H_{found}$ 2.84; purity: 99.60%.

2.1.14. 9-(2'-chloro-6'-fluorophenyl)-2,6,7-trihydroxy-xanthene-3-one (12)

Crude material; yield 88.5%; m.p. 186-188 °C; IR (KBr) λmax/cm−1: 3400-2500 (phenol OH); 860 (substituted benzene); 1600 (C=O); 1300 (C-O); 1200(def.vibr. OH); 800(C-Cl); 1100(C-F); ^{13}C NMR (150 MHz, DMSO-d_6, δ/ppm): 164.0 (C-12, C-13), 159.3 (C-2', d, $J_{C,F}$ = 242.3 Hz), 151.7 (C-2, C-7), 148.3 (C-3, C-6), 136.1 (C-9), 133.5 (C-6', d, $J_{C,F}$ = 3.9 Hz), 132.7 (C-4', d, $J_{C,F}$ = 9.3 Hz), 126.2 (C-5'), 120.5 (C-1', d, $J_{C,F}$ = 21.2 Hz), 115.4 (C-11, C-14), 115.3 (C-3', d, $J_{C,F}$ = 22.6 Hz), 105.3 (C-1, C-8), 102.7 (C-4, C-5); ^{1}H NMR (600 MHz, DMSO-d_6, δ/ppm): 9.60 (2H, br. s., OH), 8.81 (1H, br. s., OH), 7.76 (1H, d, H-4', *J* = 8.2 Hz), 7.69 (1H, t, H-5', *J* = 8.7 Hz), 7.58 (1H, td, H-3', J_1 = 8.3 Hz, J_2 = 6.1 Hz), 6.75 (2H, s, H-4,H-5), 6.22 (2H, s, H-1, H-8); ESI-MS (m/z): 373.3 $(M+H)^+$ Elemental analysis for $C_{19}H_{10}O_5FCl$ (372.5): $\%C_{calculated}$. 61.21; $\%H_{calculated}$. 2.68; $\%C_{found}$: 61.35; $\%H_{found}$ 2.72; purity: 99.82%.

2.2. Biological activity

2.2.1. Screening of the Antiplatelet Activity Induced by AA

Aggregometer Multiplate (Roche, Switzerland) was used for screening of the antiplatelet activity. This device has two pairs of electrodes submerged in stirred human blood samples to measure the impedance between them. [17] 300 µL of the whole blood was diluted by equipotent volume of pre-heated 0.9% sodium chloride and incubated with 5 µL of a tested compound dissolved in DMSO for 3 min at 37 °C. Platelet aggregation was then induced by addition of AA and the aggregation process was observed for 6 min and expressed as area under the curve. Since there is inherent variability in the general human population, the dose of AA was gradually increased in order to construct dose-response curves and to find the lowest dose to evoke maximal platelet aggregation. In screening experiments, 2 donors with comparable response to AA (113 and 117 µM) were used and other head-to-head experiments were always performed with the same donor to control for variance between individuals. ASA and kaempferol were used as standards to ensure the reproducibility of the results. All samples were compared to blank which contained 5 µL of DMSO instead of the tested compound. The concentration of DMSO was maintained in all samples at 0.8% (V/V).

2.2.2. Cyclooxygenase-1 Inhibition

A commercial set [18] which does not give false positive results for antioxidants, was used for the evaluation of COX-1 inhibition. Shortly, ASA or xanthene-3-ones dissolved in DMSO were incubated with ovine COX-1 at 37°C and AA (final concentration of 100 µM) was added to the mixture to start the reaction. The formed prostaglandin H_2 was measured following its reduction to prostaglandin $F_{2\alpha}$ by stannous chloride and assessed by enzyme immunoassay. The percentage of inhibition was related to the positive control with DMSO. In additional experiments, PRP with a platelet concentration of 3,5 $x10^8$ per mL, pre-treated with 1-benzylimidazole, to block further metabolism of prostaglandin H_2, was used instead of ovine COX-1 for testing the inhibition of human COX-1. The results were compared to ASA, the clinically used COX blocker.

2.2.3. Thromboxane A_2 Synthase Inhibition

Thromboxane A_2 synthase inhibition was evaluated according to the method of Chang, *et al* [19] with minor modifications. PRP containing indomethacin with a platelet concentration of 3.5 $x10^8$ per mL was incubated with the tested compounds for 3 min at 37 °C. After addition of prostaglandin H_2 (50 ng), the mixture was incubated for 5 min. The incubation was immediately terminated by addition of chilled EDTA (2 mM) and the solution was centrifuged at 10,500 g for 2 min (centrifuge MPW-52, MPW Med. Instruments). The thromboxane B_2 levels in the supernatant were measured using a thromboxane B_2 EIA kit according to the instructions of the manufactures. [20] Results were compared to 1-benzylimidazol, a known blocker of thromboxane A_2 synthase.

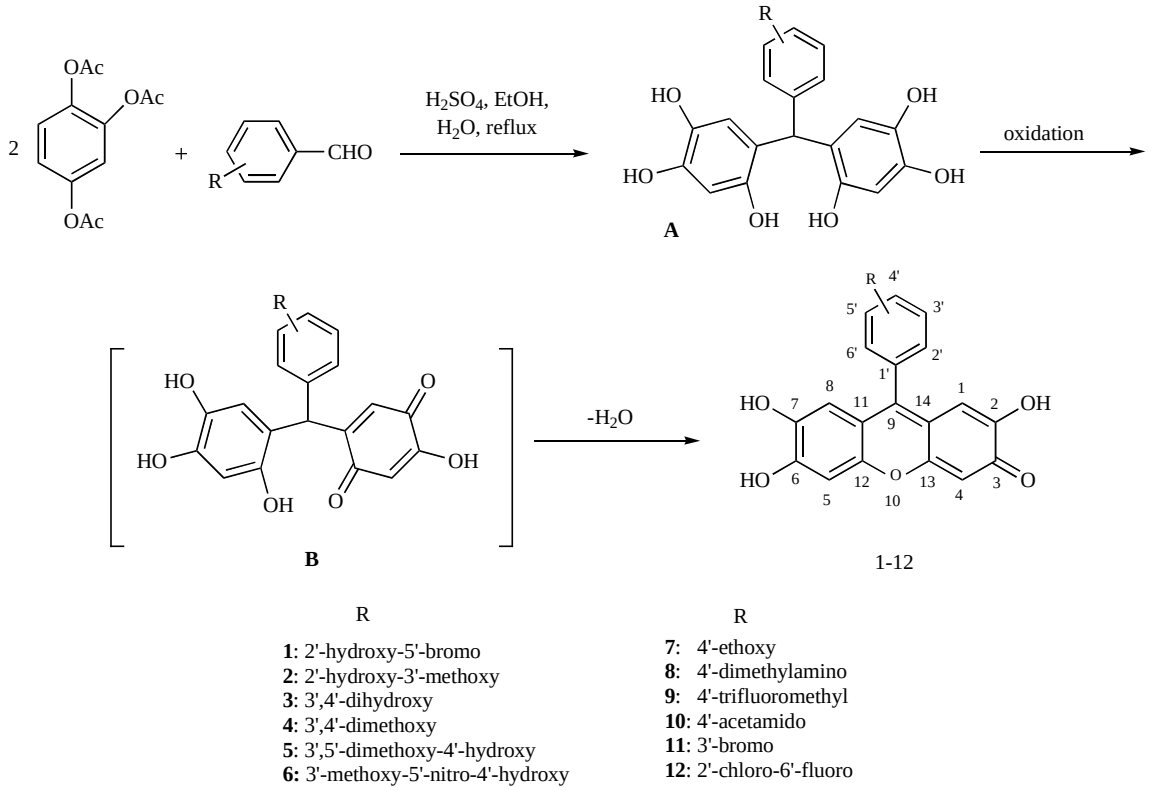

Fig. (1). Synthesis of xanthene-3-on derivatives.

2.2.4. Antagonism at the Thromboxane Receptors

Antagonism at thromboxane A_2 receptors was analysed using of the aggregometer Multiplate. The procedure was similar to the screening but the aggregation was induced by the addition of U-46619, a stable agonist of thromboxane A_2 receptors. The aggregation process was monitored for 6 min.

2.2.5. Aggregation Induced by Collagen

The approach was very similar to antiplatelet screening with AA. The only exception was that collagen was used instead of AA.

2.2.6. Statistical Analysis

Data are presented as means ± SD. The differences between compounds were assessed by one-way ANOVA followed by Tukey multiple comparison test. The differences between concentration-response curves were analysed using the 95% confidence intervals. All statistical evaluation was carried out using GraphPad Prism, version 6.00 (GraphPad Software, San Diego, USA).

2.2.7. Blood Volunteers

Blood samples from 11 non-smoking volunteers were collected by venipuncture into plastic disposable syringes containing heparin sodium (170 IU/10 mL). For specific experiments, the COX inhibitor indomethacin, or the thromboxane synthase inhibitor 1-benzylimidazole, were immediately added to the collected blood to a final concentration of 10 μM. The volunteers had not taken any medication for least 14 days before the blood collection and gave written informed consent to the study which was done with the approval of the Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové (approval date: November 12, 2012) and conforms to the latest Declaration of Helsinki. Whole blood was used in screening experiments. In other experiments, platelet rich plasma was obtained as a supernatant by centrifugation of the collected blood for 10 min at 500 g (centrifuge MPW-360, MPV Med. Instruments, Poland). Platelet poor plasma was prepared by centrifugation of the remaining blood for 10 min at 2,500 g. The platelet count was determined using a BD AccuriC6 flow cytometer (BD AccuriCytometers Inc., USA) equipped with BD CF low Software and adjusted to 2.5 or 3.5 $x10^8$ platelets/mL according to the planned protocol (see above) using autologous plasma.

3. RESULTS AND DISCUSSION

3.1. Chemistry

Twelve xanthen-3-one derivatives (**1-12**, Fig. **1**) were prepared from 1,2,4-triacetoxybenzene and different aromatic aldehydes under acidic alcoholic conditions. After a two-fold Friedel-Crafts alkylation intermediate **A** was obtained. For accomplishing the transformation, a single trihydroxy benzene moiety of **A** had to be oxidized using potassium peroxodisulphate to the corresponding *p*-benzoquinone. To avoid decomposition of potassium peroxodisulphate, the reaction of oxidation occurred at 80°C. Benzoquinone intermediate (**B**) subsequently underwent a cyclocondensation reaction to the xanthenone fragment. To remove potassium peroxodisulphate after completed oxidation, refluxed suspension was poured onto ice water and filtered. The residue was dried under vacuum at 60 °C. The synthetic pathway (**1-**

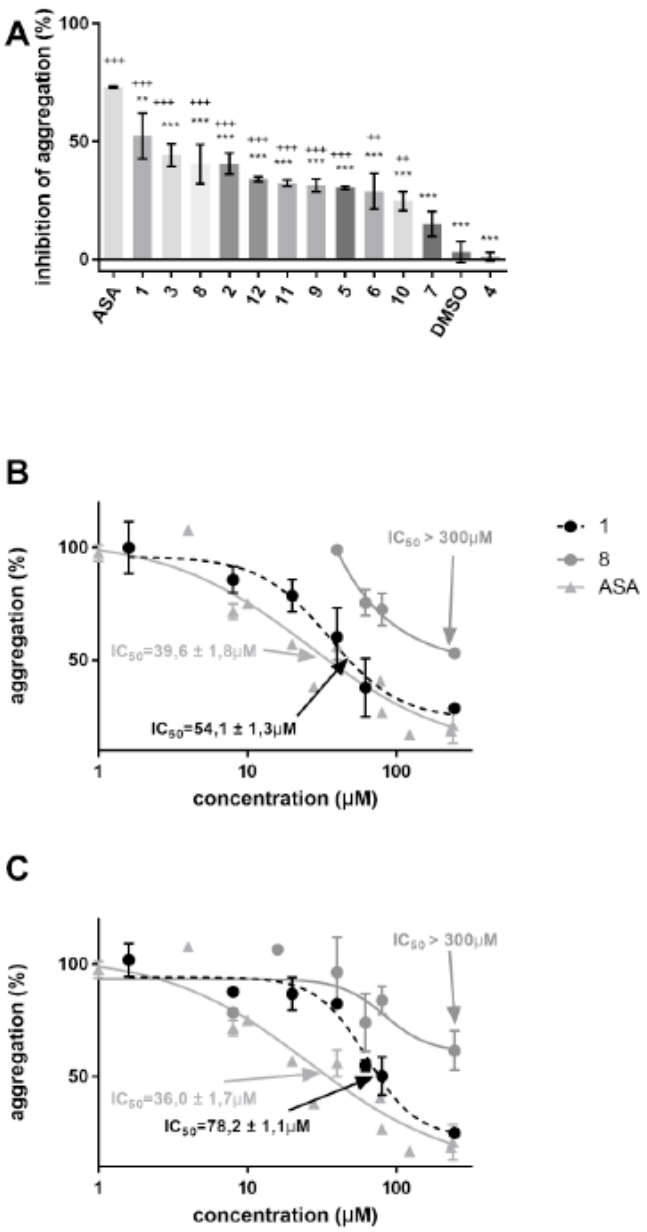

Fig. (2). Effect of compounds on whole human blood platelet aggregation induced by AA. **A**: Comparison of the effects of all tested compounds (a final concentration of 80 μM) on platelet aggregation (data from 2 blood donors, the precise final concentration of AA was 115 ± 2 μM). Concentration-response curves of two active compounds at a final concentration of 123 μM of AA (**B**) and 172 μM (**C**) for one donor. Data are presented as means ± SD. * p < 0.05; ** p < 0.01; *** p < 0.001 vs ASA; + p < 0.05; ++ p < 0.01; +++ p < 0.001 *vs.* solvent (DMSO).

12), adapted from [7] is shown in Fig. (**1**). The IR spectra of three new synthesized compounds showed absorption at 2400-3600 cm^{-1} indicating phenyl OH groups. Xanthen-3-one derivative with a methoxy group at aryl substituent (**4-6**) showed bands at 1250 cm^{-1} due to stretching vibrations of O-CH_3. The bands at 1500 cm^{-1} were visible on the spectra of the derivative with nitro group (**6**) due to stretching vibrations of C–NO_2. The compound with ethoxy group (**7**) showed absorption at 1270 cm^{-1} due to stretching vibrations of R-O-C_2H_5. On the IR spectra of compound **11** with bromine at aryl substituent were visible bands at 750 cm^{-1} due to stretching vibrations of C-Br, while compound with fluor (**9, 12**) showed bands at 1030-1100 cm^{-1} due to stretching vibrations of C-F. The IR spectra of new synthesized compounds showed absorption frequencies around 1660 cm^{-1} and 1200 cm^{-1} due to stretching vibrations of C=O and phenyl groups, respectively. On ^{1}H NMR spectra two singlet signals between 6.22 and 6.97 ppm corresponded to 4 protons from the xanthen-3-one ring. Compounds with *metha* substituted phenyl ring by methoxy group (**2, 5, 6)** on ^{1}H NMR revealed signals at 3.92 or 3.71ppm. ^{1}H NMR of compound **7** with an ethoxy group substituted on the phenyl ring revealed quartet at 4.17 ppm and triplet at 1.41 ppm. Compound **8** with a dimethylamino group on the phenyl ring on ^{1}H NMR showed singlet at 3.10 ppm. Compound **10** with acetamido group substituted on the phenyl ring at the *para* positions on ^{1}H NMR showed a doublet signal at 7.02 ppm. Characteristic signals of xanthen-3-one ring as well as all signals of substituent were found in ^{13}C NMR spectra of all synthesized compounds. Compounds with methoxy substituent (**2,5,6**) on ^{13}C NMR spectra showed signals at 55.9, 56.2 or 57 ppm associated with carbon atom from methoxy group. ^{13}C NMR spectra of ethoxy substituted derivative **7** revealed signals at 63.3 and 14.6 ppm. Compound **8** with dimethylamino group showed a signal at 39.8 ppm, while compound **9** with a trifluormethyl group substituted on the phenyl ring revealed a signal at 124.1 ppm. The results of the elemental analysis and mass spectroscopy indicate that the xanthen-3-one derivatives with described substitutions were synthesized.

3.2. Biology

3.2.1. Screening of the Antiplatelet Activity Induced by AA

At first, all the compounds were separately incubated in whole human blood to investigate any possible antiplatelet activity. Blood was induced to aggregate by the addition of arachidonic acid. ASA, the standard antiplatelet drug, was used for comparison. Almost all compounds evoked significant platelet inhibition at the highest tested concentration of 80 μM (Fig. **2A**). Additional testing with different concentrations led to the selection of two active compounds 9-(2'-hydroxy-5-bromophenyl)-2,6,7-trihydroxy-xanthen-3-one (**1**) and 9-(4'-dimethylaminophenyl)-2,6,7-trihydroxy-xanthen-3-one (**8**) for further analysis. Their concentration-response curves and at that for ASA were constructed at two different concentrations of AA in the blood of one donor to eliminate the interindividual variability (Fig. **2 B,C**). Compound **1** was a potent inhibitor of AA-induced aggregation but with significantly less effect than ASA (p<0.05). In contrast to ASA, compound **1** behaved in a competitive manner, since higher concentration of AA resulted in a decrease in antiplatelet potential (IC_{50} = 54.1 ± 1.3μM; 78.2 ± 1,1μM respectively). Compound **8** had a weaker effect independent of AA concentration used.

3.2.2. Cyclooxygenase-1 Inhibition

COX-1 inhibition was investigated as the first step in the AA based pathway to predict the mechanism of action of the selected active compounds. Compound **1** achieved higher COX-1 inhibition than ASA, whereas **8** demonstrated no effect in the test with recombinant ovine enzyme (p<0.05, Fig. **3A**). To confirm the effect in human conditions, addi-

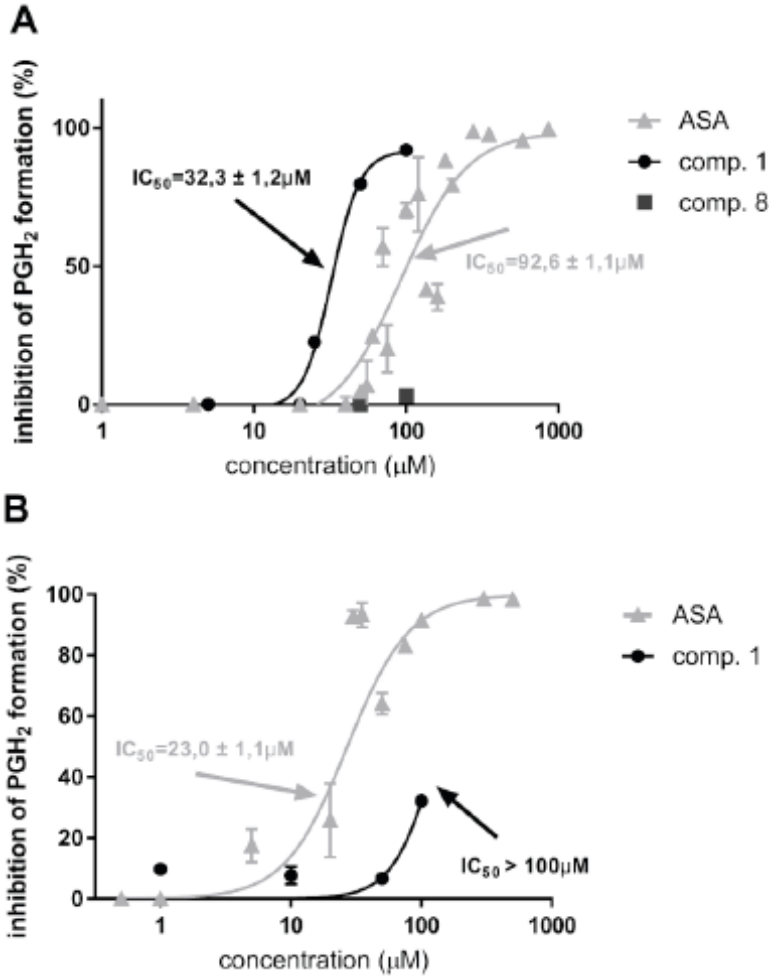

Fig. (3). Effect of tested compounds on cyclooxygenase-1. **A**: Concentration-response curves of active compound **1**, **8** and ASA on inhibition of recombinant ovine COX-1 and inhibition of human platelet COX-1 in human plasma (**B**).

tional testing was carried out on human platelet rich plasma (PRP). Here, ASA, the standard antiplatelet drug, showed excellent effect in units of μM (IC_{50} = 23.0 ±1,1 μM), while compound **1** showed no inhibition of COX-1 in human platelets in concentrations below 50 μM suggesting that this mechanism is likely unimportant for antiplatelet effects observed in full human blood (Fig. **3B**).

2.2.3. TXA_2 Inhibition

The second step of the AA cascade is the transformation of prostaglandin H_2 to TXA_2 by thromboxane A_2 synthase. None of the selected xanthene-3-ones inhibited TXA_2 production significantly at the highest tested concentration of 100 μM and are thus are considered inactive toward this enzyme (Fig. **4**).

2.2.4. Antagonism at Thromboxane Receptors

Since the effect of both tested compounds on the synthesis of both prostaglandin H_2 and thromboxane A_2 was negligible, they should have an effect upstroke in the aggregation cascade. Hence, their effect on aggregation induced by U 46619, a stable agonist at thromboxane receptors, was examined. Indeed, both compounds showed antagonism which was markedly more expressed in the case of compound **8** ($p<0.05$, Fig. **5**).

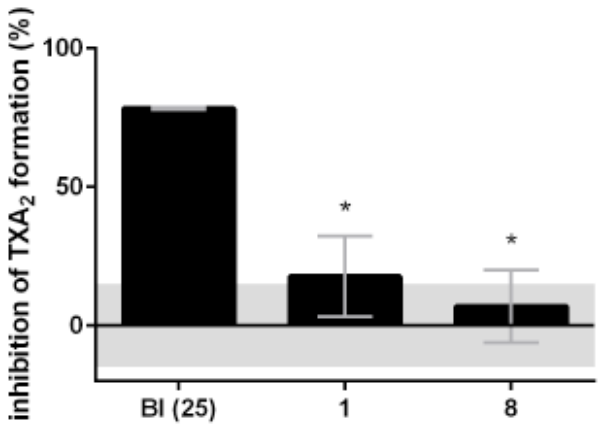

Fig. (4). Effect of active tested compounds and 1-benzylimidazole on thromboxane A_2 synthase. Compounds were tested at a concentration of 100 μM. 1-benzylimidazole at a concentration of 25 μM (BI 25) is displayed for comparison. Data are presented as means ± SD. *$p < 0.01$ vs 1-benzylimidazole. Grey area refers to the error of the method.

2.2.5. Aggregation Induced by Addition of Collagen

For confirmation of the antiplatelet properties of the two active compounds, collagen as a more physiological ex-

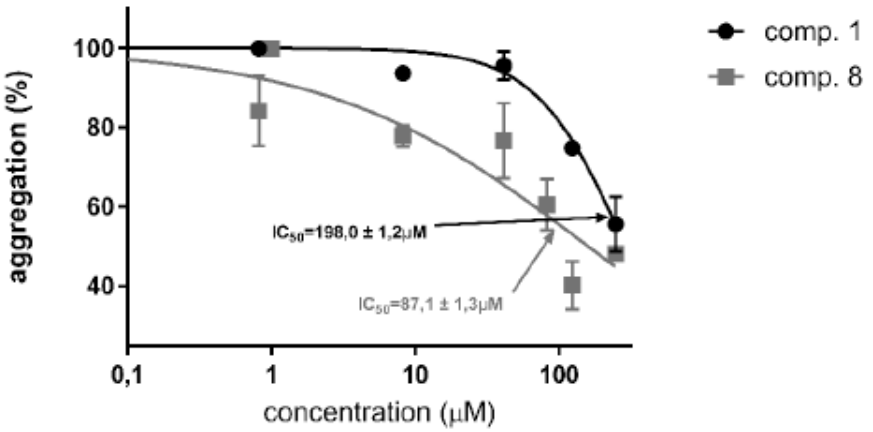

Fig. (5). Effect of compounds at thromboxane receptors. The concentrations-response curves of tested compounds on platelet aggregation induced by U-46619. Data are presented as means ± SD.

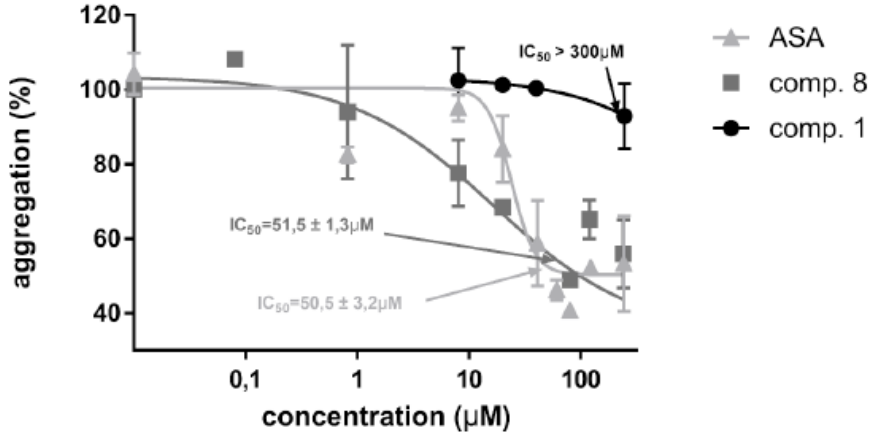

Fig. (6). Effect of compounds on whole blood platelet aggregation induced by collagen. Concentration-response curves of the two most active compounds. Data are presented as means ± SD.

tracellular inducer of platelet aggregation in whole blood was selected. The effect of compound **8** was not inferior to that of ASA, moreover this xanthene had effect in lower concentration than ASA, but due to the distinct curves their IC_{50} were comparable (51,5 ± 1,3 μM vs. 50,5 ± 3,2 μM). Compound **1** was clearly less active (Fig. **6**).

2.3. Discussion

9-aryl-2,6,7-trihydroxy-xanthen-3-one derivatives were synthesized from 1,2,4-triacetoxybenzene and different substituted aromatic aldehydes using potassium peroxydisulphate as oxidant. Best reaction conditions were obtained when the oxidant was added dropwise over a range of about 50 minutes. Additional reflux completed the conversion of benzoquinone intermediate and provided strongly coloured xanthen-3-one derivatives. Using analytical methods (elemental analyses, IR, NMR, MS) the composition and structure of the synthesized compounds were confirmed.

On the basis of the measured data, the structure-activity relationship in the group of new 9-fenylderivatives of xanthene-3-one was assessed. The most active compound blocking the aggregation induced by addition of AA was compound **1**, which has 2'-hydroxy-5'-bromophenyl group attached to the common 9-phenyl-2,6,7-trihydroxy-xanthen-3-one core. In general, the presence of a hydroxyl group in position 2´ or 3´ appears to be advantageous since beyond compound **1**, compounds **2** and **3** were also among the most active derivatives. Alkyloxy groups in position 4´ markedly reduced the effect. In particular, compound **4** with 3´,4´-dimethoxy group was inactive while compound **7** with a 4´-ethoxygroup showed weak activity. In contrast, a methoxy group in position 3´ influenced the activity only slightly (*e.g.* difference among compounds **3-5**). Different halogen substitutions, with the possible exception of bromide in position 5´ of compound **1**, had only minor effect on the activity. Direct comparison of the substituent at position 4´ showed the following order of activity: dimethylamine > trifluormethyl > acetamide > ethoxyl.

The differences in AA assay among compounds **2, 3** and **8** were very small. We selected compound **8** for further testing because it retained its effect at lower concentration (*data not shown*). This was a particularly "lucky" selection since compound **8** (4´-dimethylamino derivative) was surprisingly more active on collagen-induced aggregation (Fig. **6**) than compound **1** while the latter was the most potent inhibitor of AA-induced aggregation (Fig. **1**). The collagen pathway is more complex and the clinically used acetylsalicylic acid is more potent inhibitor of AA pathway than collagen induced aggregation [21]. Since the collagen induced pathway is associated with TxA_2, it is not surprising that a drug antagoniz-

ing the effect of TxA_2 would also block collagen-induced aggregation**.** Why compound **1** was a more potent inhibitor of platelet activation caused by AA than compound **8** even if the latter seemed to be a more potent antagonist at thromboxane receptors, is not clear. We cannot rule out the idea that compound **8** might possess an effect on the collagen pathway which was not discovered in this study. In any event, from a clinical viewpoint, a potent effect on collagen triggered platelet aggregation is more important since exogenous administration of AA is rather artificial in contrast to collagen. In sum, compound **8** had a comparable or even better effect than clinically used acetylsalicylic acid and a different mechanism of action. Since antiplatelet treatment is aimed at inhibition of platelet aggregation in among others, patients with coronary artery disease [22] and thromboxane A_2 receptors are upregulated in acute myocardial infarction [5], a (novel) compound acting as an antagonist at these thromboxane receptors could be a potentially interesting drug.

We also showed that compound **1** had very good effect in blocking recombinant ovine COX-1, but this effect was not seen in human plasma suggesting it is not important for the final effect of this compound. Some xanthene-9-ones acted as potent inhibitors of ovine cyclooxygenase 1, but due to marked structural differences, no direct conclusion can be drawn [14]. Interestingly, compound **8** did not block ovine COX-1 and no significant activity of our active compounds on thromboxane synthase was observed.

Our results suggest that future testing should be aimed in two directions: 1) *in vivo* confirmation of the antiplatelet potential of compound **8** associated with analysis of pharmacokinetics and 2) modification of compounds **1** and **8** to find the most active substitution pattern.

CONCLUSION

In summary, this study refers to a series of novel synthesized compounds with potent antiplatelet activity. The antiplatelet activity was based on antagonism of thromboxane A_2 effect. The most active compound 9-(4'-dimethylaminophenyl)-2,6,7-trihydroxy-xanthene-3-one is worthy of further *in vivo* testing of both a pharmacokinetic and pharmacodynamics, character.

LIST OF ABBREVIATIONS

AA	=	arachidonic acid
ASA	=	acetylsalicylic acid
COX-1	=	cyclooxigenase-1
EDTA	=	ethylendiamintetraacetic acid
ESI-MS	=	Electrospray ionization mass spectrometry
PRP	=	platelet rich plasma
PPP	=	platelet poor plasma
TXA_2	=	thromboxane A_2

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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